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Letter



# Environmental Surveillance of Flood Control Infrastructure Impacted by Unsheltered Individuals Leads to the Detection of SARS-CoV-2 and Novel Mutations in the Spike Gene

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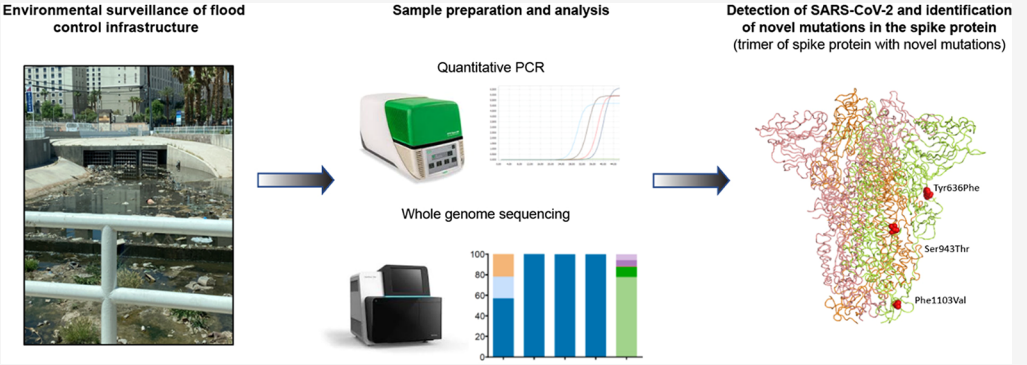


**ABSTRACT:** In the United States, the growing number of people experiencing homelessness has become a socioeconomic crisis with public health ramifications, recently exacerbated by the COVID-19 pandemic. We hypothesized that the environmental surveillance of flood control infrastructure may be an effective approach to understand the prevalence of infectious disease. From December 2021 through July 2022, we tested for SARS-CoV-2 RNA from two flood control channels known to be impacted by unsheltered individuals residing in upstream tunnels. Using qPCR, we detected SARS-CoV-2 RNA in these environmental water samples when significant COVID-19 outbreaks were occurring in the surrounding community. We also performed whole genome sequencing to identify SARS-CoV-2 lineages. Variant compositions were consistent with those of geographically and temporally matched municipal wastewater samples and clinical specimens. However, we also detected 10 of 22 mutations specific to the Alpha variant in the environmental water samples collected during January 2022—one year after the Alpha infection peak. We also identified mutations in the spike gene that have never been identified in published reports. Our findings demonstrate that environmental surveillance of flood control infrastructure may be an effective tool to understand public health conditions among unsheltered individuals—a vulnerable population that is underrepresented in clinical surveillance data.

**KEYWORDS:** *SARS-CoV-2, COVID-19, Stormwater, Wastewater, Spike, Cryptic mutations*



## INTRODUCTION

When coronavirus disease 2019 (COVID-19) was declared a public health emergency by the World Health Organization in 2020, public health laboratories in the United States (U.S.) played a pivotal role in determining case infection levels. However, these laboratories were soon overwhelmed by surveillance activities that were required to quantify and track emerging SARS-CoV-2 variants. In response to this challenge, the U.S. Centers for Disease Control and Prevention (CDC) initiated a National Wastewater Surveillance System (NWSS) to complement more traditional public health surveillance efforts.[1] SARS-CoV-2 viral RNA can be found in human feces and urine, enabling investigators to monitor wastewater and provide estimates of COVID-19 incidence or community prevalence.[2] Since March 2020, we and others have demonstrated that wastewater surveillance can provide an early detection system for high-priority pathogens, antimicrobial resistance markers, high risk substances, and emerging SARS-CoV-2 variants.[3−15]

In addition to reporting on SARS-CoV-2 viral RNA in wastewater, several studies have described the detection of viral material and human fecal contamination in flood control

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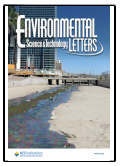

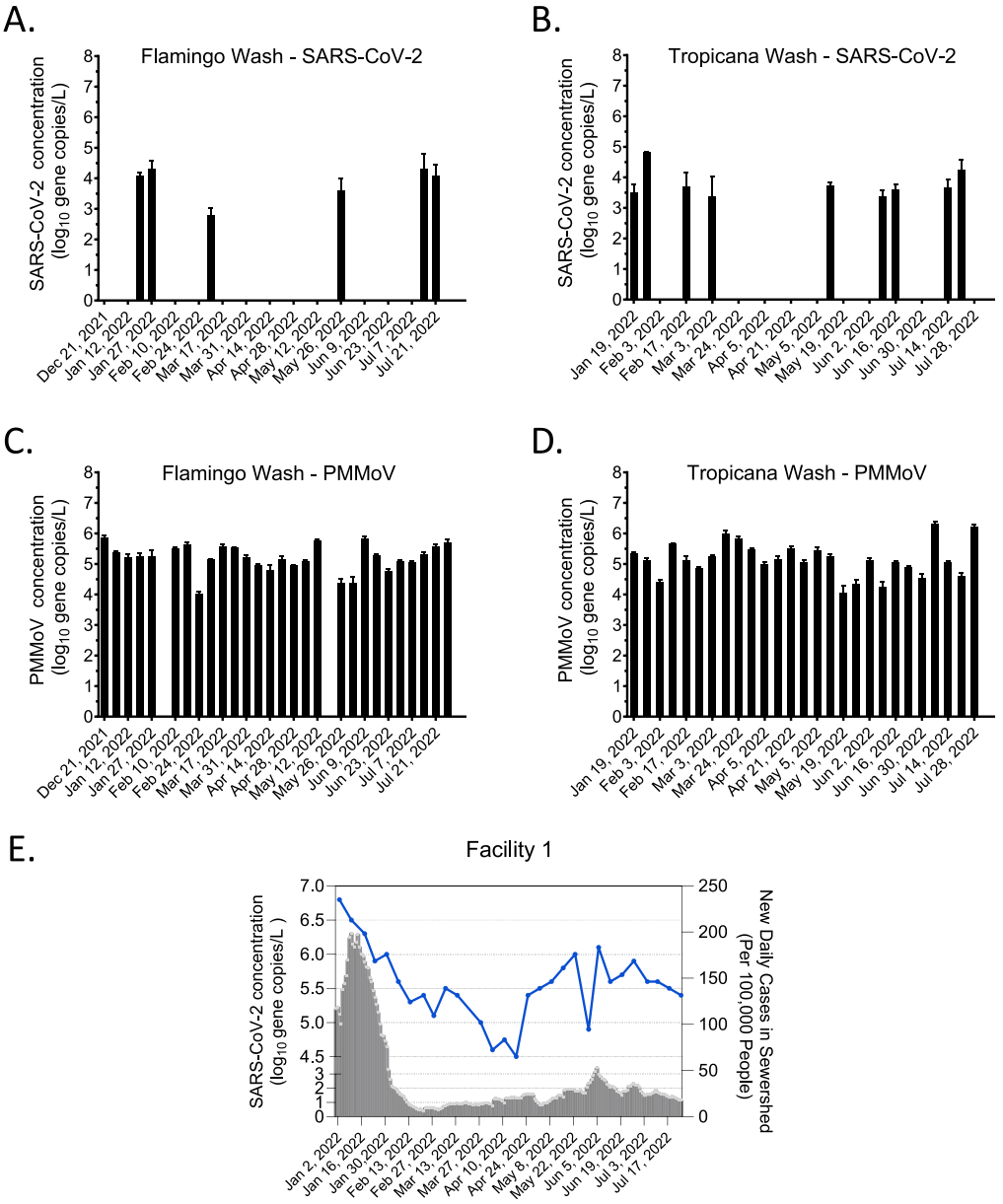


**Figure 1.** Characterization of SARS-CoV-2 and PMMoV RNA concentrations in environmental water samples (no recovery adjustment) and municipal wastewater samples (with recovery adjustment) from the Las Vegas Metropolitan Area. (A, B) SARS-CoV-2 concentrations and (C, D) PMMoV concentrations (both in $\log_{10}$ gene copies/L) in environmental water samples collected from Flamingo Wash (FW) and Tropicana Wash (TW) from December 2021 to July 2022. The absence of bars for SARS-CoV-2 or PMMoV indicates no detection of the target. (E) Wastewater SARS-CoV-2 concentrations in $\log_{10}$ gc/L (blue line) and sewershed-specific confirmed case counts per 100,000 people (gray bars) for the wastewater treatment plant (WWTP) in this study.

infrastructure due to overflowing sanitary sewers, sewer collection system leaks, and direct human inputs.[16−18] In cities like Las Vegas, environmental factors (e.g., relatively mild winters) coupled with city ordinances criminalizing unsheltered homelessness have led to the establishment of encampments in remote areas, including flood control channels, where makeshift toilets and open defecation are common.[18] These unsheltered individuals are at higher risk of infection, morbidity, and disease-associated mortality because of their limited access to health care services.[19−21] Thus, wastewater surveillance of homeless shelters[8] and environmental surveillance of flood control infrastructure may help characterize public health conditions and disease transmission, including for COVID-19, within this particularly vulnerable population that is often underrepresented in traditional clinical surveillance data.

SARS-CoV-2 viral RNA can persist for up to several weeks in environmental waters, and potentially longer depending on the temperature of the water.[22] In addition, SARS-CoV-2 infected individuals shed maximum viral RNA in fecal matter at the onset of infection and up to seven months after the initial infection.[8,22−24] While previous studies on SARS-CoV-2 in surface waters, including stormwater, have documented SARS-CoV-2 RNA levels by quantitative polymerase chain reaction (qPCR),[16,17] whole genome sequencing (WGS) of samples from flood control channels (e.g., urban runoff and stormwater) to generate SARS-CoV-2 variant information is less common.[16,17,25] This could be due to challenges with


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collection and analysis of these complex samples, specifically performing WGS on low viral loads or in the presence of unique PCR inhibitors which negatively influence the success of sequencing workflows.[7,26] Obtaining knowledge about the SARS-CoV-2 variant composition in urban runoff and stormwater could support public health surveillance of new reservoirs of viral variants. Therefore, the objectives of this study were to (1) determine if SARS-CoV-2 RNA could be detected in environmental water samples collected from flood control infrastructure that is known to be impacted by unsheltered individuals, (2) conduct amplicon-based WGS of SARS-CoV-2 from these environmental water samples, (3) compare SARS-CoV-2 variants present in the environmental water samples with those circulating in the local community (via wastewater and clinical surveillance), and (4) investigate if novel mutations could be detected.

## METHODS

**Sample Collection, Processing, and Analysis.** Sample processing and RT-qPCR analysis for the WWTP samples followed our previously published protocols.[6,8] Briefly, 10 L of grab primary effluent were concentrated by hollow fiber ultrafiltration (HFUF) (REXEED-25S, 30 kDa, Asahi Kasei Medical Co., Japan), and the HFUF concentrate was centrifuged at 3500*g* for 30 min to remove solids. The resulting supernatant was extracted with a PureLink Viral RNA/DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA), processed with a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and analyzed by qPCR using a Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). For the environmental water samples from TW and FW, we extracted nucleic acids from 80 mL of samples using the Wizard Enviro TNA Kit (Promega) according to the manufacturer's instructions, and we eluted the nucleic acids in 50 $\mu$L of RNase-free $dH_2O$. First-strand cDNA was synthesized using the LunaScript RT SuperMix Kit (New England BioLabs). Quantification of SARS-CoV-2 RNA from the FW and TW samples was performed by using a Bio-Rad Opus 96 Real-Time PCR instrument. Additional details are included in the Supporting Information (Text S1).

**Library Preparation and Sequencing.** Tailed amplicon-based sequencing libraries were constructed using the CleanPlex SARS-CoV-2 FLEX Panel from Paragon Genomics according to the manufacturer's instructions. More than 10 ng of total RNA was processed for first-strand cDNA synthesis (LunaScript RT SuperMix Kit, New England BioLabs). Samples were sequenced weekly or biweekly using an Illumina NextSeq 500 sequencer with a midoutput v2.5 (300 cycles) flow cell.[8,27] Raw fastq files are available on the National Center for Biotechnology Information (NCBI) website under BioProject PRJNA886745.

**Analysis of Sequencing Data.** Illumina adapter sequences were trimmed using cutadapt (v3.2). All sequencing reads were mapped to the SARS-CoV-2 genome (NC_045512.2) using bwa-mem (v0.7.17-r1188). Amplicon primers were masked from aligned reads using fgbio TrimPrimers (version 1.3.0), and variants were called using iVar variants (v1.3). Genome coverages were calculated by Samtools (v1.10). SARS-CoV-2 variant composition was determined using Freyja (v1.3.10).[28] Sam Refiner (v1.4) identified low frequency and novel mutations that were validated by searching outbreak.info, Nextstrain, and GISAID databases.[29−32]

**Human Subjects and Ethics Statement.** The University of Nevada Las Vegas Institutional Review Board reviewed this project and determined it to be exempt from human subject research according to federal regulations and university policy. While reports of infections, or concentrations of infectious disease targets in environmental samples, may have the potential to stigmatize groups of individuals,[33−35] this public health surveillance effort preserved individual anonymity at all times. Throughout the development and implementation of our research plan, we maintained regular communication with various community stakeholders, who are experts in working with unhoused individuals.

## RESULTS AND DISCUSSION

**Detection of SARS-CoV-2 and PMMoV Viral RNA in Urban Runoff and WWTP Samples.** SARS-CoV-2 viral loads in fecal matter of infected individuals have been reported to be as high as $10^7$ to almost $10^{10}$ gene copies (gc) per gram or mL of feces,[36,37] with some wastewater studies supporting these high end estimates.[8] Therefore, particularly in systems with minimal dilution, such as direct human inputs into low-flow environmental systems (e.g., urban runoff), even a single infected individual has the potential to cause high SARS-COV-2 RNA concentrations.

Out of 57 samples obtained from TW and FW, 15 samples contained detectable levels of SARS-CoV-2 RNA (detection frequencies of 33% for TW and 20% for FW), and concentrations (not adjusted for recovery) ranged from 2.8 to 4.8 $\log_{10}$ gc/L (Figure 1A−D). For TW and to a lesser extent FW, SARS-CoV-2 RNA was detected at a higher frequency at the beginning of the study (i.e., January and February 2022; Figure 1A−D), which corresponded with the peak of the first Omicron wave (i.e., BA.1). In the Las Vegas Metropolitan Area, this initial Omicron wave resulted in the highest observed concentrations at the WWTP to date, reaching a recovery-adjusted peak of 6.8 $\log_{10}$ gc/L (Figure 1E), and approximately 20% of the local population being infected based on a wastewater-derived incidence estimate.[10] Even clinical testing data, which have become increasingly biased by the public's transition to rapid at-home tests, indicated approximately 200 new daily cases per 100,000 residents within the sewershed during this time (Figure 1E). With this high level of COVID-19 incidence and prevalence across the local population, it is plausible that similar levels of transmission were occurring among sheltered and unsheltered individuals experiencing homelessness.

As a proxy for human fecal material, we also tested for PMMoV and detected the RNA of this fecal indicator virus in all samples except two FW samples collected on February 3 and May 19 (Figure 1**C**−**D**), with concentrations ranging from 4.0 to 6.3 $\log_{10}$ gc/L (not adjusted for recovery). These PMMoV concentrations are consistent with those reported for the same tributary washes in a previous microbial source tracking study.[18] The PMMoV detection frequencies in the current study (100% for TW and 93% for FW) are slightly higher than those in the earlier study (72%), perhaps due to greater sensitivity of the sample processing method or the closer sampling proximity to areas with higher density unsheltered individuals. For the WWTP, PMMoV was detected in 100% of samples with concentrations ranging from 8.2 to 8.8 $\log_{10}$ gc/L (not adjusted for recovery) or 8.2 to 9.1 $\log_{10}$ gc/L (adjusted for BCoV recovery) (data not shown in Figure 1).



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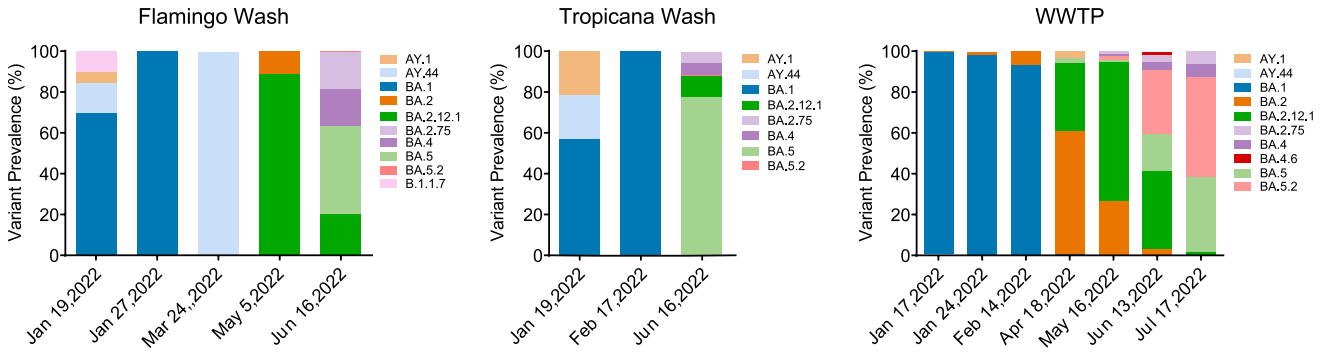


**Figure 2.** Variant prevalence in environmental water samples (i.e., Flamingo Wash and Tropicana Wash) and wastewater treatment plant (WWTP) samples, as determined by WGS with the following sequencing thresholds: >80% genome coverage at >100-fold sequencing depth.

**SARS-CoV-2 Whole Genome Sequencing (WGS) Variant Composition in Urban Runoff and WWTP Samples.** Environmental water samples may contain low SARS-CoV-2 content overall and/or fragmented viral RNA.[38−40] In response to working with degraded RNA, amplicon-based WGS has been one approach for generating SARS-CoV-2 sequencing libraries. In contrast to clinical samples from individuals, wastewater samples contain many different pooled SARS-CoV-2 genomes, which makes haplotype phasing and estimating variant abundances challenging. Recently, an important method for estimating variant abundances was developed.[28] This bioinformatics tool, called Freyja, can provide an estimation of SARS-CoV-2 variant abundances by applying a depth-weighted least absolute deviation regression analysis.[28] Low-quality sequence data and a lack of established criteria for analysis are current challenges for WGS of environmental samples, leading to sequencing depth being a critical variable influencing the accuracy of variant annotation. Interestingly, the developers of Freyja analyzed wastewater samples that yielded >50%−75% genome coverage, but did not discuss a minimum sequencing depth threshold. This standard may be the result of their method assigning more priority to variant-defining mutations with greater sequencing depth. In another study involving mixed sample deconvolution, a sequencing depth of 1000X was proposed as a minimum threshold, but the authors did not discuss a minimum value for genome coverage.[41] Different sequencing technologies (Illumina and Oxford Nanopore), sequencing instruments, and library preparation kits can be used for SARS-CoV-2 WGS, and each of these variables can impact sequencing metrics.

Given that the recommended sequencing criteria for clinical samples is >90% genome coverage at >100X sequencing depth, we decided to use similar criteria for this study and only analyzed samples that had >80% genome coverage at >100X sequencing depth (Supplementary Table 1 and Supplementary Table 2).[38,42] During this study, Freyja classified SARS-CoV-2 variants in the WWTP and environmental water samples predominantly as Omicron, but earlier variants, specifically Delta (AY.1 and AY.44) and to a lesser extent Alpha (B.1.1.7), were also predicted and to a greater extent in the environmental water samples (Figure 2). The Alpha variant (B.1.1.7) was classified at a low proportion (10.4%) in FW on January 19, 2022 (Figure 2), while the last reported Alpha variant infection in Nevada (EPI_ISL_5163539) was four months earlier in September 2021. To verify the Alpha lineage designation by the Freyja software tool, we manually inspected each of Alpha's characteristic mutations. Out of the 22 mutations found in the Alpha lineage,[43,44] we detected 10 mutations in the FW sample indicating a good but imperfect match (Supplementary Table 3). The time discrepancy for Alpha detection could suggest that direct human inputs into FW, perhaps by one or more unsheltered individuals, were linked to persistent shedding or to low circulation levels of the Alpha variant. In fact, a recent study of New York wastewater indicates that persistent shedding is associated with cryptic lineages that differ from prevailing variants.[45] The Delta variant (AY.1 and AY.44) was predicted at both sites but at relatively low proportions on January 19, 2022 (Figure 2). More than two months later, on March 24, 2022, well after Omicron had established itself as the dominant variant in the local community, AY.44 accounted for ∼100% of the genomes in FW (Figure 2). According to GISAID, the last reported Delta variant infection in Nevada was in February 2022 (EPI_ISL_9916131). In contrast with the Alpha example, detecting a Delta variant signal in the environmental water samples is within the shedding timeline for a typical SARS-CoV-2 infection. Moreover, because of the presumably variable and intermittent nature of the loadings to these environmental sites (i.e., relative to a municipal wastewater), it is possible that the signal from one infected individual could drastically shift the composition of the environmental water samples toward an unexpected variant.

The relative abundance of Omicron subvariants in the environmental water samples was similar to that of the geographically matched WWTP. For example, during the month of May 2022, samples from both FW and the WWTP were dominated by BA.2.12.1, and in the month of June, all sites showed BA.5 increasing in abundance (Figure 2). Overall, these results demonstrate that environmental water samples from flood control channels impacted by unsheltered individuals appear to contain the same SARS-CoV-2 variants circulating in the broader community (i.e., at the WWTP), with some intermittent signals from previously circulating variants.

**Novel Mutations of SARS-CoV-2 Spike Protein.** SARS-CoV-2 wastewater surveillance in New York City led to the discovery of novel mutations of the spike protein using targeted amplicon sequencing.[46] Some of those novel mutations were found in seven wastewater WGS data sets from the same time frame. These cryptic lineages were hypothesized to come from unsampled COVID-19 infections in the area or from an animal reservoir.[46] To screen for potential novel mutations in the environmental water samples


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and WWTP samples from Las Vegas, we applied sequencing metrics of >80% genome coverage at >100X sequencing depth. Using this threshold, we found three novel mutations (Tyr636Phe, Ser943Thr, and Phe1103Val) that were observed more than once (Supplementary Table 4). The novel mutation with the highest detection frequency was Tyr636Phe, which was observed at TW and FW on one date in January, only at TW on two dates in February, and only at the WWTP several months later, in July 2022. Another mutation, Phe1103Val, was detected at both environmental sites on one date in January and only at TW on one date in February. TW also had one novel mutation specific to its site, Ser943Thr, which was observed once in January and once in February. Due to the mutations not residing in the spike protein receptor-binding domain (RBD), we did not conduct investigations into the receptor-binding effects that they may have on SARS-CoV-2 function. Whether these mutations belong to a cryptic lineage unique to the Las Vegas flood control channel or municipal wastewater remains to be determined, but the fact that Tyr636Phe was observed at both environmental sites and at the WWTP suggests that this mutation may be associated with a SARS-CoV-2 variant circulating in the broader local community (i.e., may or may not be linked to unsheltered individuals) (Supplementary Table 4). It is also possible that a single individual could be contributing to both systems, although a rare variant is less likely to be detected in a system as large as the WWTP in this study.

In summary, we determined that SARS-CoV-2 variants circulating in the local community (i.e., via wastewater or clinical surveillance) were generally the same variants that dominated environmental water samples from flood control channels that were presumably impacted by human waste from unsheltered individuals.[18] SARS-CoV-2 RNA concentrations in the environmental water samples were highest during the peak of the initial Omicron surge (i.e., BA.1), which was consistent with the concentrations detected at the WWTP and confirmed case counts from clinical surveillance. These initial high-frequency detections of SARS-CoV-2 RNA in environmental water samples were followed by a period of frequent nondetects, which was correlated with a rapid decline in WWTP concentrations and confirmed case counts. Assuming that detections in the environmental water samples were linked to unsheltered individuals, this suggests that COVID-19 transmission within this disadvantaged population mirrored that of the broader community. We communicated these findings to our local and state public health officials and to local homeless resource and recovery centers and service providers. Due to a lack of clinical surveillance data for unsheltered individuals, we were unable to directly compare variant prevalence for the environmental water samples with this particular population.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.3c00938.

Additional experimental methods, figures of sampling locations, tables with sequencing metrics and mutations for samples, and relevant references (PDF)

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### Author Contributions

▽Anthony Harrington and Van Vo contributed equally to this article.


### Notes

The authors declare no competing financial interest.

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